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2,3-DPG-Hb complex: a hypothesis for an asy binding

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Abstract

This study was undertaken to test the symmetry of 2,3-diphosphoglycerate (2,3-DPG) binding site in hemoglobin (Hb). From Arnone's study [A. Arnone, Nature (London) 237 (1972) 146] the 2,3-DPG binding site is located at the top of the cavity, that runs through the center of the deoxy-Hb molecule. However, it is possible that this symmetry reported by Arnone, for crystals of 2,3-DPG-Hb complex, might not be conserved in solution. In this paper, we report the ^{31}P nuclear magnetic resonances of the 2,3-DPG interaction with Hb. The 2,3-DPG chemical shifts of the P_2 and P_3 resonance are both pH- and hemoglobin-dependent [protein from man, polar bear (*Ursus maritimus*), Arctic fox (*Alopex lagopus*) and bovine]. 2,3-DPG binds tightly to deoxyhemoglobin and weakly, nevertheless significantly, to oxyhemoglobin. In particular, our results suggest similar spatial position of the binding site of 2,3-DPG in both forms of Hb in solutions. However, the most unexpected result was the apparent loss of symmetry in the binding site, which might correlate with the ability of the hemoglobin to modulate its functional behavior. The different interactions of the phosphate groups indicate small differences in the quaternary structure of the different deoxy forms of hemoglobin. Given the above structural perturbation an asymmetric binding in the complex could justify, at least in part, different physiological properties of Hb. Regardless, functionally relevant effects of 2,3-DPG seem to be measured and best elucidated through solution studies.

Keywords: Hemoglobin; 2,3-DPG-Hb complex; HbA; Polar bear Hb; Arctic fox Hb; Bovine Hb; ^{31}P-NMR

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1. Introduction

Mammalian erythrocytes contain 2,3-DPG in about equimolar proportion to Hb. The organophosphate 2,3-DPG increased the partial pressures of oxygen needed to produce half saturation with oxygen. Under physiological conditions of pH and ionic strength, 2,3-DPG associates especially with deoxyhemoglobin (in the ratio of 1 mol per mol tetramer [1–3]), in a cleft between the two β chains with following shifting of the equilibrium affinity curve to the right. Perutz [4] placed 2,3-DPG in the central cavity of the deoxy model (T form), and coordinated by VAL β1, HIS β2, LYS β82 and HIS β143, that are within hydrogen-binding distance and are complementary to the anionic charges of 2,3-DPG. In particular, on transition to the quaternary oxy structure (R form), the valines move apart and the helices H move together so that the stereochemical complementary of the binding site is destroyed and the 2,3-DPG drops out. Because the affinity of 2,3-DPG for Hb is lowered by salt, showing that the binding is electrostatic and is totally inhibited at the salt concentrations used to precipitate crystals of deoxyhemoglobin, these findings suggest the presence, in solution, of some important differences with the precedent Hb-2,3-DPG high-salt structure solved by Arnone [5]. From the analysis of this author [5], in the T state the central cavity of human Hb becomes greater and the allosteric effector 2,3-DPG binds to the protein by non-covalent, electrostatic interactions. The first questions, at this point, might be how much greater is the cavity, when in solution, and is the enlargement proportional to the loss of O_2 affinity. A second question pertains to the symmetry of this enlargement, considering that 2,3-DPG is an asymmetric molecule. However, considering the crystal symmetrical structure of human deoxyhemoglobin A complexed with 2,3-DPG, an additional question is, how could the symmetry cause an asymmetric effect. Finally, the pK of the positively charged groups facing 2,3-DPG depends on the distance among positively and negatively charged groups. In human Hb, the span between α-amino residues growths from 16 Å to 20 Å, so that they can no longer interact with the phosphate groups of 2,3-DPG, and consequently their pK decreases. 2,3-DPG stabilizes the quaternary deoxy structure cross-linking the β chains, which probably does not directly effect the tertiary structure. However, hydrogen ions influence both the tertiary and the quaternary structures by stabilizing the deoxy form. Nevertheless, if the influence of H^+ ions is graduated, compatible with the co-operative effect, the distance between the α-amino groups of the cavity would increase as the pH moves towards alkaline values. However, decreasing the fraction of the α-amino residues carrying positive charges would decrease the electrostatic interaction between them and the 2,3-DPG charges. It is possible that the only difference is that the binding site in oxyhemoglobin involves fewer interactions and this differential binding of 2,3-DPG to oxy- and deoxyhemoglobin would be sufficient to explain its role as a metabolic effector of the oxygen affinity of Hb. On purpose, the ^{31}P nuclear magnetic resonance (NMR), which is very sensitive to changes in chemical environment, has been used.

In this study, the authors report, and comment on, ^{31}P NMR resonances of the 2,3-DPG interaction with various hemoglobins (human, bovine, polar bear and Arctic fox) in solution.

2. Materials and methods

Unless otherwise stated, all chemicals employed were obtained in the highest grade commercially available from Aldrich Co.

2.1. Protein preparation

Human blood was sampled from one of the authors (C.B.). Human red cells were lysed with two volumes of 1 mM Tris–HCl buffer (pH 8.0); stromas were eliminated by centrifugation. Stripped hemoglobin was obtained by passing the hemolysate over a mixed-bed ion-exchange column (Bio-Rad AG 501-X8) or a Sephadex G-25 column (2–4°) equilibrated with 10 mM Tris–HCl (pH 8), containing 0.1 M NaCl [6]. Electrophoretic analysis was performed on cellulose acetate at pH 9.2. The concentration in

aliquots was adjusted to 0.3 g/d and 0.025 ml was applied for electrophoresis, 30 min at 350 V, 4°C using Tris–EDTA–glycine buffer. All the Hb samples used in the following resonance experiments showed only one major component (HbA). Samples for NMR spectra were prepared according to the general procedure.

Bovine blood samples, from the local slaughterhouse, were worked out with the same procedure reported for human Hb. The electrophoretic mobility, on cellulose acetate, shows that bovine Hb co-migrates with human Hb A at alkaline pH. These experiments showed only one major component; this is an important result because most species of European (and American) cows are heterozygous [7], but this was not the case. The electrophoretic analysis was also performed by electrophoresis in sodium dodecyl sulfate-polyacrylamide (SDS–PAGE, pH 8.3 buffer Tris–Glycine) according to Laemmli [8]. The purified bovine Hb samples at concentration of 60 mg/ml were used in the following NMR experiments.

Polar bear (*Ursus maritimus*), blood samples, from Svalbard (NO), were collected by A.E.D. and Ø.W., into an isotonic NaCl solution containing 2 mM EDTA. Peripheral blood cells were washed four to five times in 1.2% NaCl before a volume of distilled water, equal to the packed cell volume, was added. Lysis was completed by freeze-thawing. Stromas were eliminated by centrifugation. Removal of any organic phosphates from bear Hb was obtained by gel filtration on a Sephadex G-25 column equilibrated with 10 mM Tris–HCl (pH 8), at temperature 2–4°C. Identification of the Hb components was determined by electrophoretic mobility on cellulose acetate; also in this case the hemoglobin co-migrated with human Hb A at alkaline pH. These experiments showed only one major component. The purified Hb samples at concentration of 3–4 g/100 ml were used in the following NMR experiments.

Arctic fox (*Alopex lagopus*) blood, also from Svalbard, was collected by E.F.; samples were prepared as reported for bear Hb. Also in this case, on cellulose acetate the electrophoretic mobility (pH 9.2) showed one component. Ringed seal hemoglobins, which were well separated in two distinct spots, were used as reference. The purified Hb samples, at concentration of ≈ 3 g/100 ml, were used in the following NMR experiments. Even in this case, only one hemoglobin was present which presented an electrophoretic mobility near the one of the polar bear Hb.

Methemoglobin was checked in all experiments by measuring the absorption at 630 nm and found to be less than 2%.

2.2. Nuclear magnetic resonance

Two different procedures were performed. In the first procedure, samples were prepared by gel filtration (Sephadex G25) and subsequent mixed-bed ion-exchange resin (Bio-Rad AG 501-X8) [6]. Solutions for ^{31}P-NMR were made up in 95% H_2O/5% D_2O and contained ≈ 0.4 mM deoxy-Hb (tetramer), 8 mM sodium dithionite, ≈ 0.4 mM 2,3-DPG, final buffer concentration 100 mM HEPES (pH range 6.5–8.0) or MES (pH < 6.5). The NMR spectra were recorded by a Varian Gemini spectrometer operating at 121.48 MHz for ^{31}P. However, even if reproducible, these samples at the end of the resonance experiment presented dramatic amounts of methemoglobin and broad signals. In particular the value of pK of the P_2 phosphate group was different from that obtained from the vacuum procedure (Δp$K \approx 0.6$ pH units). For all these reasons we decided to avoid the use of dithionite. In the second approach the deoxygenation was achieved by alternate exposure to vacuum with gentle agitation to expose the maximum surface area; under this conditions, foaming is minimized; NMR tube were filled by means of water-saturated nitrogen and sealed. In all the experiments the goodness of fit was guaranteed: $R^2 \geq 0.99$; absolute sum of squares: $0.01 \div 0.0003$; pK S.E.: $0.05 \div 0.007$. Given the results obtained, this procedure was used throughout the study. The ^{31}P spectrum was in this case the result of averaging $600 \div 400$ transients at a repetition rate of 0.7 s^{-1}. All ^{31}P chemical shifts are referenced with respect to 85% H_3PO_4. In the NMR experiments, in the presence of buffer 0.1 M, the ratio 2,3-DPG/Hb is 1:1. Concentration of Hb in NMR experiments

was ≈30 mg/ml. The temperature inside the probe was 20°C. Chemical shifts (δ) are relative to that of H_3PO_4 ($\delta_{Cn\text{-}P}-\delta_{H_3PO_4}$) and in ppm, parts per million. The δ scale is presently defined as positive in the low-field direction. The assignment of the resonance to the 3-phosphate (lower field) and to the 2-phosphate (higher field) is taken from Moon and Richards [9].

3. Results

Fig. 1 shows two high resolution NMR spectra for 2,3-DPG complexed with polar bear Hb. The corresponding chemical shifts (spectra not shown) for the resonances of DPG, in absence of protein, were 4.21 (P_3) and 3.26 (P_2), and 4.01 (P_3) and 2.99 (P_2) for pH 7.55 and pH 7.40, respectively; hence these spectra show a significant interaction between the phosphate groups, P_2 and P_3, of the organic effector and the protein. Figs. 2 and 3 display the ^{31}P NMR data for the chemical shifts of P_2 and P_3 phosphate groups of 2,3-DPG molecule. Very similar ^{31}P titration of Hb A were reported by Russu et al. [10]. Unexpectedly, the pH downfield shift of these phosphate groups, with reference to the free 2,3-DPG, gave similar results for all hemoglobins. In fact, the absence of two charges in the 2,3-DPG binding-site decreased the affinity of 2,3-DPG for fetal Hb; however, this was not the case for bovine Hb which, apparently, strongly reacts with the phosphate effector (Table 1).

In Table 1, the values of the first two columns (relative to the deoxyhemoglobin-2,3-DPG complex) are directly proportional to the interaction of the 2,3-DPG molecule in the binding site of the protein. In particular, even if the central cavity in the oxyhemoglobin is closed, 2,3-DPG is still interacting with Hb. It is also possible for 2,3-DPG be inside the cavity, but with a weaker interaction (and a weaker binding constant). The increasing of the distance between the α-amino groups does not mean that 2,3-DPG can no longer react by hydrogen bonds with the amino group of one of the two lysines which, among the residues of the cationic cavity, are most able to follow the eventual shift of the 2,3-DPG molecule inside the cavity. The values of the last two columns in

Table 1
Difference, Δδ (ppm), for P_2 and P_3 chemical shift (δ) relative to: (deoxyhemoglobin-2,3-DPG complex)−(free 2,3-DPG); (deoxy−oxy) complex[a]

Hb	Complex−free		Deoxy−oxy	
	Δδ P_2	Δδ P_3	Δδ P_2	Δδ P_3
Man	1.45	0.8	0.9	0.6
Bovine	1.35	0.65	0.5	≈0
Polar bear	1.35	0.6	0.8	0.2
Arctic fox	1.25	0.6	0.7	0.45

[a] Hepes buffer 0.1 M (pH 7.4); temperature 20°C.

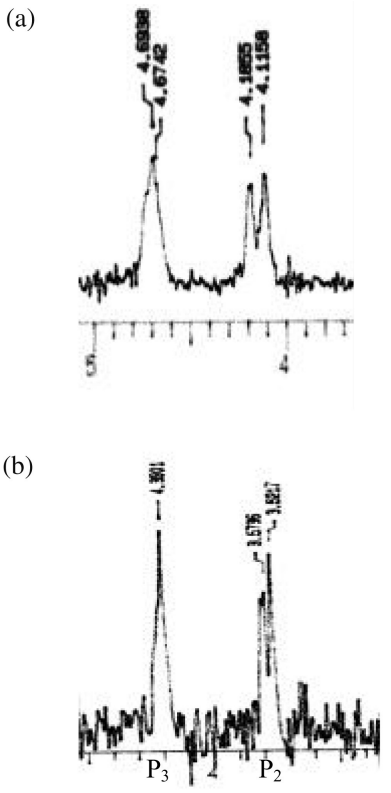

Fig. 1. Polar bear hemoglobin (≈1:1 molar ratio to 2,3-DPG): 121.48 MHz ^{31}P-NMR spectra of the 2- and 3-phosphate groups of 2,3-diphosphoglycerate in 0.1 M MES buffer at 20°C: (A) pH 7.55 deoxy-Hb (≈30 mg/ml); (B) pH 7.40 oxy-Hb (≈50 mg/ml). The assignment of the resonances to the 3-phosphate (lower field) and to the 2-phosphate (higher field) is taken from Moon and Richards [9]. Absolute shifts are from external H_3PO_4.

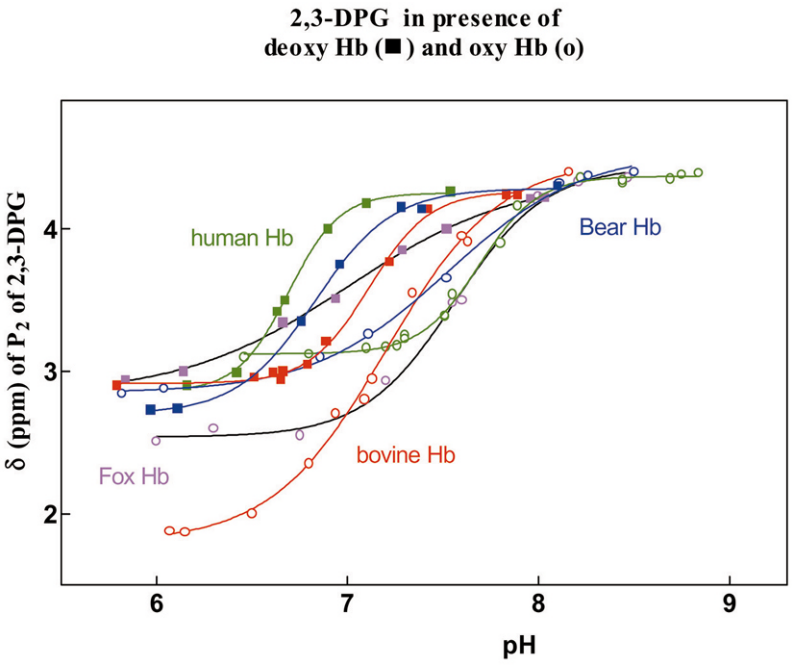

Fig. 2. The pH-dependent chemical shift behavior (^{31}P-NMR) of P_2 of 2,3-diphosphoglycerate-Hb complex for human, bovine, polar bear and Arctic fox hemoglobins. Concentration of Hb ≈ 30 ÷ 60 mg/ml. Hb samples at various pH-values were obtained by the addition of 1.0 M MES (pH range 5.5–6.5) or of 1.0 M HEPES (pH range 6.5–8.0). The final buffer concentration was 0.1 M. The temperature inside the probe was 20°C. Chemical shifts (δ) are relative to that of H_3PO_4 ($\delta_{Cn\text{-}P}-\delta_{H_3PO_4}$). p$K$ S.E.: 0.05 ÷ 0.009.

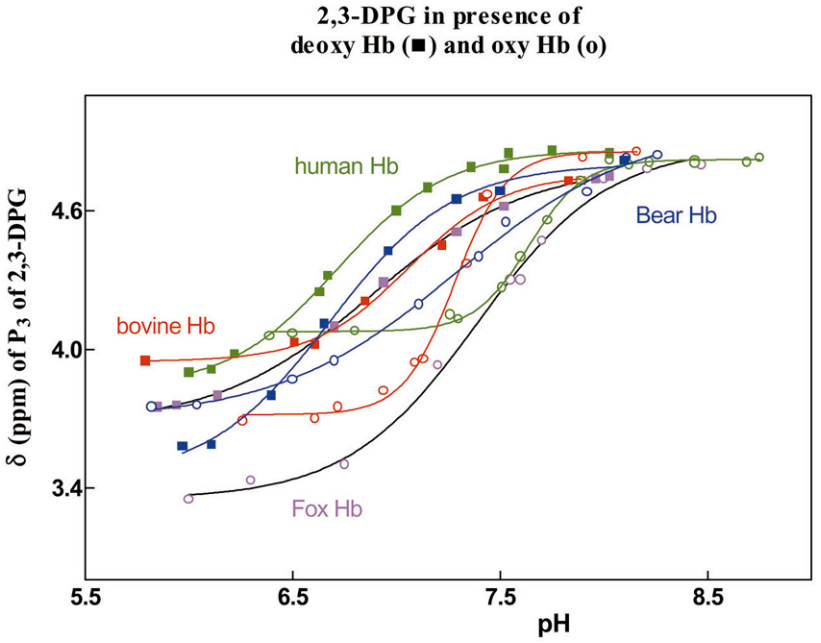

Fig. 3. The pH-dependent chemical shift behavior (^{31}P-NMR) of P_3 of 2,3-diphosphoglycerate-Hb complex for human, bovine, polar bear and Arctic fox hemoglobins. Experimental conditions as in Fig. 1. pK S.E.: 0.02 ÷ 0.007.

Table 1, are indicative of the different intensity of the interaction between the deoxy and the oxy forms of the corresponding complex with 2,3-DPG-Hb. However, if we compare the P_3 chemical shifts in the fourth column obtained for bovine Hb we can observe that the difference between these deoxy end oxy forms, under the conditions considered, is the lowest value. This result indicates that this second phosphate group of 2,3-DPG reacts with the deoxy protein site by a much weaker interaction. Nevertheless, the second column of Table 1 shows that this interaction for the deoxy form of bovine Hb was very similar to the interaction that operates in the corresponding state of human Hb. Strong interactions which operate on P_3 in oxy Hbs (bovine > bear > human $\approx$ fox, at pH 7.4) can produce this effect. Moreover, for P_3 group, a different interaction is also present in the deoxy state of these Hbs (human > bear $\geq$ bovine $\geq$ fox, at pH 7.4) which suggests an asymmetric binding in the deoxyhemoglobin-2,3-DPG complex. Furthermore, Figs. 2 and 3 show that the effect of pH on P_3 values is smaller than on P_2 atom (Table 2). Consequently, the rise in pK_as of the cationic groups of the 2,3-DPG cleft must be very different among these Hbs. Hence, either the cavity cannot be symmetric or the phosphate group of P_2 and P_3 interact with very different groups. But this last hypothesis is less likely because all data shows the correspondence of the residues involved (only in bovine Hb the replacement in the β chains of VAL1-HIS2 by MET alone reduces the number of interactions operative in the complex). Therefore, this effect could depend on the asymmetric interactions between the globins and the two phosphates groups. If the two phosphates are not equivalent for the protein, how could the resulting complex be symmetrical? The local dyad axis that relates $\alpha_1\beta_1$ and $\alpha_2\beta_2$ subunits runs, in Arnone's study [5], through the 2,3-DPG binding site. In the presence of a symmetrical electron density, evidently found by this author, the 2,3-DPG molecule was interpreted to bind to the β subunits in two different, but symmetrical, orientations. On the other hand, the acid group of 2,3-DPG might be frozen in only one of the two possible positions and, consequently, bound to only one of the two LYS 82. But in this case how can we justify the results obtained by Arnone in his study? The crystals used by Arnone were grown from concentrated ammonium sulfate solution and binding can be totally inhibited at the salt concentration (ionic strength about 7 M) used to precipitate the crystals of deoxyhemoglobin [11]. In agreement with these considerations, Richard et al. [12] reported X-ray analysis of the 2,3-DPG complex obtained using crystals grown from a low-salt solution. Surprisingly, they found that the 2,3-DPG molecule was bound to the Hb tetramer in only one orientation showing the existence of an unexpected asymmetry of the binding site. In addition, very recently, Fang et al. [13] have reported that pK values for β143HIS are quite low (4.7 and 5.6 in deoxy and CO forms of Hb A, respectively). Given the situation, the salt bridge between β143HIS and 2,3-DPG should be very weak in the neutral pH range. Table 1 adds additional evidence on the asymmetry of the binding cavity; in fact, also for human deoxyhemoglobin P_3 interaction results over 55% lower (pH 7.4) than the corresponding P_2 interaction. Quite interesting the parallel value for the other hemoglobins is about 50%. Considering these facts, even if before binding 2,3-DPG the cavity was symmetric (but this aspect depends on the role of the solvent molecules), it seems to become asymmetric after binding, as results from the chemical shifts of P_2 and P_3. Hence, within all these hemoglobins, there is a weaker P_3 interaction operating; this weaker interaction may correlate with the relative widening of the central cavity.

In addition, if the binding is highly symmetric the chemical shifts, of both phosphorous atoms P_2

Table 2
Slope, $\partial\delta$ (ppm)/ ∂pH, for P_2 and P_3 chemical shift (δ) relative to deoxyhemoglobin-2,3-DPG complex (data from Figs. 2 and 3)

	$\partial\delta P_2/\partial pH$	$\partial\delta P_3/\partial pH$
2,3-DPG alone	1.6	1.3
Man	4.0	0.9
Polar bear	4.0	0.8
Bovine	4.0	0.7
Arctic fox	0.7	0.7

and P_3, when plotted vs. pH, and for different Hb, are expected to change in a very related, if not very similar, partnership. However, as shown in Figs. 2 and 3, this was not the case.

The importance of the NMR data in define the type of interaction between 2,3-DPG and Hb was also evident for interpretation of affinity results. In fact, measures of the O_2 affinity in the presence of 2,3-DPG for both human and bovine Hb show, quite surprisingly, very similar values [14]. From these data we could, erroneously, deduce that 2,3-DPG is binding in both Hb in the same way, while Figs. 2 and 3 show a great difference in chemical shifts (that is in binding) for P_2 and P_3.

Table 2 shows, for the complexes listed, different slopes obtained for the graphs reported in Figs. 2 and 3. Whatever the slope of the lines on this graphs, however, it is always true that any slope reproduced the effect of pH on the ratio conjugate base/conjugate acid. However, the higher this ratio is, in the range of pH considered, the lower the pK is [this effect is proportional to the ratio activity (A^-)/activity (HA) which, in turn, is proportional to K/activity (H^+)]. It follows from this consideration that the pK relative to P_2 phosphate group is increasing and, conversely, is decreasing for P_3 group; with the only exception of fox Hb complex. In the case of fox Hb, P_2 and P_3 groups seem to form (Table 2) weaker salt-bridges cross-linking the β subunits. At the same time, as the interaction results weaker, the incidental enlargement of the cavity would favor the intervention of the hydrogen-bonded water molecules.

4. Discussion

A recent paper by Fang et al. [13] reported pK values for β143HIS as 4.7 for deoxyhemoglobin A and 5.6 for HbCO A. Thus, under physiological pH, the binding of 2,3-DPG to β143HIS would be very weak. Hence, β143HIS was not involved in the binding of 2,3-DPG under physiological conditions. This conclusion does not agree with the crystal structure of deoxyhemoglobin A complexed with 2,3-DPG [5] in particular on the possibility that 2,3-DPG could form a sort of salt bridge with the N-terminal amino groups of the β143HIS. The present approach (^{31}P-NMR), allowed us to determine the structural and functional interactions important in solution, seems to reach the same conclusion. In fact, the chemical shifts of phosphate groups can be used to directly measure the binding of 2,3-DPG to the protein in solution and, even more interesting, at different pH-values [10]. In this regard, it is relevant, from a functional perspective, to consider the superimposition of the values obtained for deoxy and oxyhemoglobin at alkaline pH: Figs. 2 and 3 show that 2,3-DPG interacts with a very similar aminoacidic environment. These results led us to conclude that the binding site for 2,3-DPG to oxyhemoglobin is in the same part of the Hb molecule as the deoxyhemoglobin binding site, that is at the interface of the two β-chains. During oxygenation some residues would remain charged (for example, the lysines in the central cavity) which are still effective for interacting with 2,3-DPG.

However, this does not seem to be true in the range of physiological pH (Figs. 2 and 3). A major finding of our study was that there was evidence of the presence of asymmetric binding (Figs. 2 and 3) of the effector 2,3-DPG in the central cavity of Hb. These results, in particular, raise a number of questions. First, is this asymmetry indicative of the order in which the subunits α_1, α_2, β_1, and β_2 react? According to the results at alkaline pH, if the 2,3-DPG molecule lies in the same cavity both in oxy- and deoxyhemoglobin, it is possible that the ββ cross-linking by the phosphoric effector will modulate the precedence, of one subunit (α) over the other (β), as a function of the asymmetric reversible binding between 2,3-DPG and Hb. Unfortunately, we cannot use our results to deduce kinetic observations. If the 2,3-DPG molecule binds to only one of the two LYS 82β (hence in only one alignment of the two possible orientations related by a 180° degree rotation around the dyad axis) this situation could resolve in an enlargement of the cavity.

Furthermore, if our model is bovine deoxyhemoglobin, where the chemical shift observed results from a widening of the 2,3-DPG cavity, the same mechanism (decrease of effector–protein

interaction = widening of the cavity) might be true, in solution, even for oxyhemoglobin. A wider cavity would imply the loss of the stereochemical complementarity due to non-symmetric action either of the effector or of the solvent molecules. In this case, the results described herein contrast with some previous studies [15,16] which suggest that on transition from R to the T structure there is a widening of the central cavity. In particular the behavioral effect of the P_3 group led us to conclude that the asymmetry of binding would be, for the studied Hbs: fox ≥ bear ≥ bovine > human. Finally, although the interactions of 2,3-DPG in Hb A have been investigated the most intensively using X-ray crystallographic studies, recently Gottfried et al. [17] have reported, by a combination of front-face detection and lifetime measurements, that HPT, a fluorescent analogous of 2,3-DPG, binds to the central cavity of carboxyhemoglobin A.

From the results reported, it is reasonable that inside the cavity, the binding interactions would be different for different sources of the protein and therefore the symmetry could not be justified for every species, every pH and every ionic strength (see X-ray data) even if the cavity presented the same facing cationic groups, as it is the case for human, bear and fox hemoglobins. On the whole, this study might reveal the presence of an asymmetric interaction that takes place inside the 2,3-DPG binding site. Its physiological importance is easily understandable considering the influence that this asymmetry might have on the functional properties of the Hb.

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